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CHEMOTACTIC FACTOR PRODUCTION BY RAT POLYMORPHONUCLEAR LEUKOCYTES: STIMULATION WITH OPSONIZED ZYMOSAN PARTICLES AND INHIBITION BY DEXAMETHASONE

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SUMMARY: Rat polymorphonuclear leukocytes exposed to opsonised zymosan particles in vitro instantaneously and continuously release a chemotactic factor in the medium. The activity of this factor was mainly attributed to leukotriene B_4, based on the data with high performance liquid chromatography. Preincubation of the cells with an antiinflammatory steroid, dexamethasone, at a dose of 0.25 μg/ml caused suppression in generation of the chemotactic factor from the leukocytes in a time-dependent manner.

Polymorphonuclear leukocytes (PMN)[1], when exposed in vitro to various inflammatory stimuli, were reported to secrete neutral proteases, to generate superoxide and to produce arachidonate metabolites (1). This suggests that PMN in the inflammatory locus play an important role in the progress of inflammation. As a lipoxygenase metabolite of arachidonate, LTB_4, is potently leukotactic (2), it seems rational to postulate a role of PMN in a positive feedback process in PMN accumulation at inflammatory sites via generation of LTB_4. For the mechanisms of inhibition of leukocyte infiltration by glucocorticoids, many reports (3-5) dealt with direct actions in vitro of the steroids on leukocyte motility, but few were concerned with influence of the steroid in vitro on possible production of chemotactic factors by the leukocytes. Therefore, the present experiments were undertaken to examine a possibilty of production of LTB_4 by PMN as a chemotactic factor, on the one hand, and to clarify, on the other hand, whether dexamethasone is capable of interfering with production of such self-chemotactic substances.

[1] Abbreviations used are; PMN: polymorphonuclear leukocytes, HPLC: high performance liquid chromatography, ZAS: zymosan-activated serum, CA index: chemotactic activity index, LDH: lactate dehydrogenase.

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MATERIALS AND METHODS

PMN preparation Male rats (Sprague-Dawley strain, specific pathogen free, weighing 300-500 g, Charles River Japan, Kanagawa, Japan), were injected intraperitoneally with 1% casein in Ca^{2+}-free Krebs-Ringer bicarbonate solution, and 14 hr later peritoneal fluids were entirely collected and centrifuged (400 x g, 5 min, 2°C). The cell pellet was washed twice with Gey's balanced salt solution, and finally suspended in RPMI-1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.2% bovine serum albumin. More than 90% of the cells were identified as PMN microscopically.

Incubation of PMN with opsonized zymosan particles The zymosan particles (Sigma Chemical Co., St. Louis, MO, USA) were opsonized by incubating them with normal rat serum (10 mg/ml) at 37°C for 30 min. Then the particles were washed twice with phosphate-buffered saline, suspended in the medium at various concentrations and added to the PMN suspension. Final concentration of PMN was adjusted to 5 x 10^6 cells/ml. The mixture was incubated for an appropriate time at 37°C in an atmosphare of O_2-CO_2 (95:5). After centrifugation (400 x g, 5 min, 2°C), the supernatant was collected and used to determine its chemotactic activity. The precipitate was smeared on glass slides and stained with hematoxylin and eosin. The zymosan particles engulfed or bound by the cells were counted microscopically and the average number of such particles per cell was used as phagocytic index.

Extraction and purification of lipophilic components from the medium The supernatant prepared as above was processed according to the method of Powell (6) to collect arachidonate metabolites with the aid of SEPPAK C18 cartridge (Waters Associates, Inc., Milford, MA, USA). Lipophilic substances retained in the cartridge were finally eluted with 5 ml of methanol. After filtration and evaporation of the methanol eluate, the residue dissolved in a minimum volume of methanol was subjected to HPLC on the reversed phase column (Zorbax ODS, 4.6 mm x 150 mm, Dupon Instruments) and eluted with methanol/ H_2O/acetic acid (75:25:0.01, v/v/v) at flow rate of 1 ml/min according to the procedure by Borgeat & Samuelsson (7). Synthetic LTB_4 (kindly supplied by ONO Pharmaceutical Co., Osaka, Japan) was also applied to HPLC as a reference standard. The eluate fractions were evaporated and the residues were dissolved in the medium by sonication for chemotaxis assay. The methanol eluate from SEPPAK C18 cartridge was also dissolved in the medium similarly for chemotaxis assay.

Chemotaxis assay The ^{51}Cr-labeling method described by Gallin et al. was used with modifications reported (8). Casein-induced PMN, which were radiolabeled with $Na_2{}^{51}CrO_4$, were applied to the upper compartment of the Boyden chamber and a sample to the bottom compartment. The chamber was incubated at 37°C for 75 min. And the migration was determined by radioactivity in the lower filter of double filter system in the chamber. The migration for 2.5% solution of ZAS was also determined and CA index of a sample was expressed as percent of the migration for 2.5% ZAS.

Treatment of PMN with dexamethasone Ethanol solution of dexamethasone (Sigma Chemical Co.) was added to the PMN suspension or mixture of PMN and zymosan to adjust the final ethanol concentration to 0.1% (v/v) and incubated at 37°C in O_2-CO_2(95:5). All the data represent the mean with SEM of 3 samples and statistical analysis was performed on F-test.

RESULTS AND DISCUSSION

Rat PMN stimulated by the opsonized zymosan particles released chemotactic activity for PMN in the medium in dependence on the concentration of the zymosan particles (Fig.1) and incubation time as well (Fig.2). PMN are

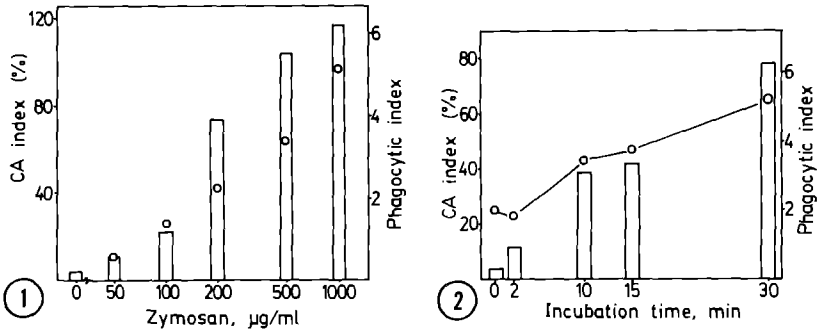

Fig.1. Stimulating effect of the zymosan particles on the production of chemotactic factor by PMN.
The opsonized zymosan particles and PMN were mixed in suspension and incubated at 37°C. Final concentration of PMN were fixed at 5 x 10^6 cells/ml and those of zymosan were varied as indicated. Chemotactic activity of the supernatant and phagocytic index of cells in the mixture are shown by columns and circles, respectively.

Fig.2. Time change of the chemotactic activity produced by the PMN exposed to zymosan.
Concentrations of zymosan and PMN in the mixture were 300 μg/ml and 5 x 10^6 cells/ml, respectively. The mixture was incubated for indicated periods. Open circles and columns show the phagocytic index and CA index, respectively.

known to stick and phagocytose opsonized zymosan particles by interactions between opsonic factors and the receptors on PMN cell membrane (9). This was observable immediately after start of the incubation (Fig.2). Generation of the chemotactic activity appeared to be triggered by such processes and the activity in the medium rose in parallel with the increase of the number of the zymosan particles combined with PMN. In the experiments for time course study (Fig.2), activity of enzymes released in the medium from PMN was also determined to correlate generation of chemotactic activity with the enzyme release. Liberation of β-glucuronidase and lysozyme which are granular components occurred later than the appearance of the chemotactic activity in the medium, while the level of LDH, a cytosol marker, in the medium was unchanged. Therefore, it is unlikely that the chemotactic activity was liberated from some intracellular storages, though some granular components like crystal-induced chemotactic factor (10) are reported.

In order to characterize chemotactic substances released, lipophilic components were extracted from the incubation medium with the aid of

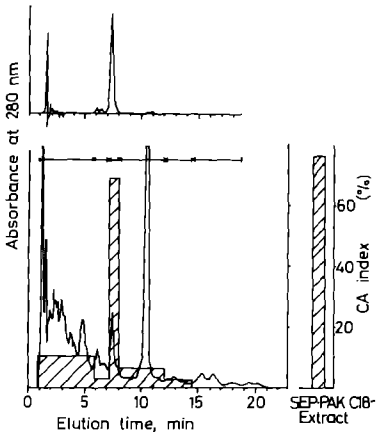

Fig.3. Isolation by HPLC of the chemotactic substance produced by PMN. The mixture of zymosan (500 μg/ml) and PMN (5 x 10^6 cells/ml) was incubated for 30 min and its supernatant was acidified and applied to SAP-PAK C18 cartridge. The methanol eluate from the cartridge was subjected to HPLC. Synthetic LTB_4 (10 ng) was also applied to HPLC.
Upper panel: Reference chromatogram of synthetic LTB_4.
Lower panel: Chromatogram of the sample. Hutched columns show the chemotactic activity of the SEP-PAK C18-extract and its eluate from HPLC, which was fractionated and pooled at intervals indicated by segments of a line with allow heads.

SEP-PAK C18 and processed by reversed phase-HPLC (Fig.3). Roughly 80% of the chemotactic activity in the incubation medium was recovered in the SEP-PAK C18-extract. Intense chemotactic activity was found exclusively in the HPLC peak eluting at a retention time identical to that of synthetic LTB_4. The CA index in the LTB_4-equivalent fraction was 69.2% and was comparable to that (76.3%) of the original SEP-PAK C18-extract on the basis of dilution to the same volume. Although the so-called LTB_4 fraction separated by a single operation using a reversed phase-HPLC might contain some of the isomers of LTB_4 (11), a strong chemotactic activity of our LTB_4 fraction seems enough for us to deduce it to be LTB_4 itself, since chemotactic activity of those isomers were reportedly very weak (11).

Dexamethasone dose-dependently inhibited the generation of chemotactic factor by PMN (Fig.4). A maximum of 65% inhibition was observed with 0.25 μg/ml (6.4 x 10^{-7}M) of dexamethasone, but even in the higher doses complete suppression was not attained. By contrast, no significant change was observed in the phagocytosis of zymosan or the activities of β-glucuronidase and LDH in the medium. Dexamethasone effectively inhibits leukocyte

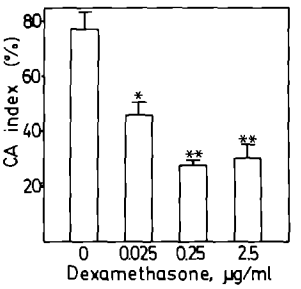

Fig.4. Inhibitory effect of various doses of dexamethasone on the generation of the chemotactic activity.
PMN were pretreated with the indicated doses of dexamethasone for 120 min at 37°C and then exposed to zymosan (500 μg/ml) for 30 min at 37°C. Dexamethasone was also present at indicated levels in the mixture of PMN and zymosan.
*:$P<0.005$, **:$P<0.001$.

infiltration at the inflammatory site even when applied at a local dose as low as 0.25 μg/ml (12). Consistently, generation of chemotactic factors attributable to LTB_4 was considerably suppressed by dexamethasone at dose levels equivalent to those effective in the experiment in vivo. Therefore, it is likely that glucocorticoids inhibit leukocyte infiltration through reduction in the production of LTB_4 at the inflammatory site.

The data summarized in Fig.5 reveal that the inhibitory effect of dexamethasone enhanced as a function of time for pretreatment of PMN. When PMN pretreated with dexamethasone was exposed, after its separation from the preincubation medium, to zymosan in a fresh medium without

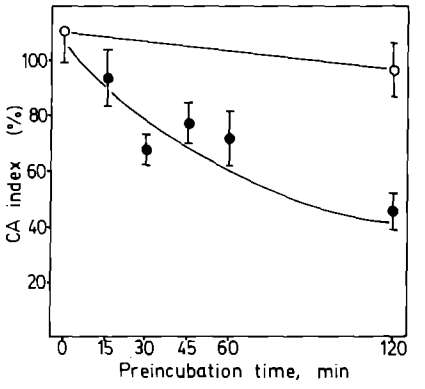

Fig.5. Time-dependent inhibitory effect of dexamethasone on the production of the chemotactic activity.
PMN were pretreated with (●) or without (○) dexamethasone (0.25 μg/ml) for indicated periods and after washing the cells were suspended with zymosan (500 μg/ml) in a fresh medium and incubated for 30 min without dexamethasone.

dexamethasone, the inhibitory effect was observed to the same extent as in the cells kept in the medium where the cells were preincubated with dexamethasone (data not shown). In contrast, the medium in which PMN were treated with dexamethasone did not interfere at all with the generation of chemotactic activity when incubated with freshly prepared PMN. Dexamethasone, when applied directly in the mixture of PMN and zymosan without pretreatment of the cells, exerted no detectable effect. Reduction by glucocorticoids of the production of arachidonate metabolites such as LTB_4 seems in general to be ascribed to production of phospholipase-inhibitory proteins (13,14). Such inhibitory proteins are reported to be secreted extracellularly from macrophases or leukocytes. The present results were somewhat different from the above concept in that release from dexamethasone-treated cells of any inhibitory factors against production of LTB_4-like substance was undetected, whereas the treated cells became less active for the production of LTB_4-like substance.

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